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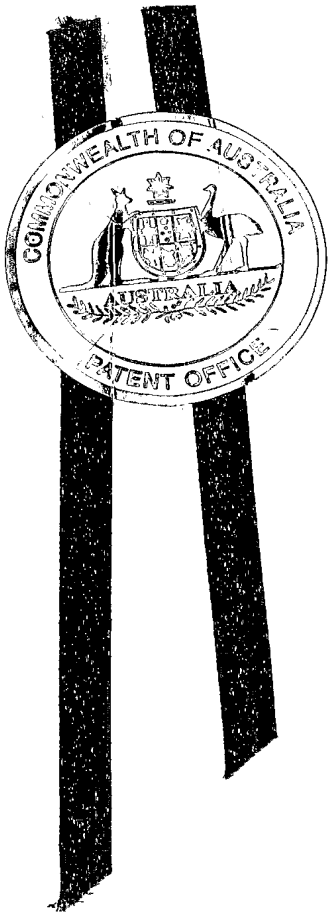


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# PROVISIONAL SPECIFICATION

for the invention entitled:

"A plant extract"

The invention is described in the following statement:

# A PLANT EXTRACT

## BACKGROUND OF THE INVENTION

### FIELD OF THE INVENTION

The present invention relates generally to plant extracts comprising components that, together and/or separately, exhibit desirable properties. More particularly, the present invention relates to extracts and components thereof from the plant genus *Zingiber* and in particular from the rhizome of the species *Zingiber officinale* (also known as ginger) which comprise activities having applicability in a wide range of useful fields. One particularly useful activity is an enzyme protease activity. This activity may exist in a single entity or within a mixture of two or more entities. Examples of the range of fields of application include, but are not limited to: use as a research reagent for specific breakdown and/or harvesting of target molecules and/or cells; pharmaceutical and/or nutraceutical product development; manufacture of improved high-value food and feed products, production of ethanol from cereals, and waste treatment *inter alia*. The present invention also provides *Zingiber* extracts such as ginger crush and dry ginger and *Zingiber* components such as Zingibain. Specific applications of the plant extracts and components of the present invention encompass, *inter alia*, food and feed processing; allergen removal and/or inactivation; blood clot prevention and/or disintegration; wound healing, and prophylaxis and/or treatment in a range of disease conditions extending to cancer, inflammatory conditions, and the inhibition of virus infection.

### DESCRIPTION OF THE PRIOR ART

Bibliographic details of the publications referred to by author in this specification are collected at the end of the description.

Reference to any prior art in this specification is not, and should not be taken as, an acknowledgment or any form of suggestion that this prior art forms part of the common general knowledge in any country.

Extracts from the tissues of monocotyledonous and dicotyledonous plants have provided a vast number of compounds and mixtures of compounds useful in medicine - including both Western-style and traditional approaches, such as those used in Sharmanism and Chinese medicine, for example; building construction; the automotive industry, and biotechnology. Particular extracts from the tissues of monocotyledonous and dicotyledonous plants are extremely useful, for instance, in the areas of food processing and food technology. In such areas, extracts made from plant tissues provide a very diverse range of additives and treatments for food, including spices, colouring, preservatives and condiments to flavour food, and compounds to treat food to increase palatability. Extracts from plant tissues also provide compounds that can be used to enhance the long-term storage capacity and shelf life of manufactured and processed food.

Globally, there is an increasing demand for agents to enhance the value of human food and animal feed products and to extract food value from processed bio-matter. Value-adding approaches include the use of agents such as plant extracts to assist in the improvement of the palatability and digestibility of protein derived from animals. Increasingly, plant extracts and compositions derived therefrom, also have applications in a range of areas related to animal and human health - from prophylaxis to diagnosis and therapy. In addition, there is increasing interest in identifying agents that are efficacious in improving the quality of food preparations, or that may be used separately or added to foods to produce a so-called "functional food". A functional food is that which, *per se*, has an identifiable quality or qualities associated with maintaining health and/or preventing deterioration thereof. An improved quality of an existing food may be, for example, a "no-fat", "salt-reduced" or "allergen-free" equivalent of an existing food item. These concepts apply equally to feed for the many animal industries as to food for human ingestion. Furthermore, plant extracts are of use in industrial applications that require, for example, a protein recovery phase, as well as in areas such as waste management.

The rhizome of the ginger plant, *Zingiber officinale*, has been used as a spice in food preparation and as a non-specific "herbal remedy" for various disease conditions. Neither the efficacy nor the underlying activity, however, has been delineated or quantified in a manner permitting reliable reproducible outcomes, sufficient for consistent treatment purposes. Furthermore, studies designed to assess such presumptions have had to contend with the over-riding difficulty of lack of consistent and reproducible trial data.

In accordance with the present invention, the difficulties associated with variability and lack of consistency of various extracts and components of the ginger rhizome have been overcome. This has enabled the quantification and characterization of the ginger rhizome and extracts thereof and its components. The inventors sought to quantify and characterize useful activities and the identification of a wide range of useful applications for the extracts and/or components thereof.

## SUMMARY OF THE INVENTION

Throughout this specification, unless the context requires otherwise, the word Acomprise≅, or variations such as Acomprises≅ or Acomprising≅, will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

Nucleotide and amino acid sequences are referred to by a sequence identifier number (SEQ ID NO:). The SEQ ID NOs: correspond numerically to the sequence identifiers <400>1 (SEQ ID NO:1), <400>2 (SEQ ID NO:2), etc. A summary of the sequence identifiers is provided in Table 1. A sequence listing is provided at the end of the specification.

The present invention provides extracts, and components thereof, derived from members of the Zingiberaceae plant family. Members of this family include, for example, *Zingiber mioga*, *Zingiber officinale*, *Zingiber cassumunar* and *Zingiber zerumbet*. The preferred species is *Zingiber officinale*, also known as ginger. The extracts and components, derived from the rhizome of the *Z. officinale* plant, comprise activities which are able to be applied usefully in a very wide range of related fields, extending from animal and human feed/food and health maintenance, to disease prophylaxis and treatment. Even more diverse areas, such as laboratory applications in the life sciences, including cell and molecular biology applications, and industrial applications such as the production of ethanol from cereals and the treatment of waste products, are contemplated, assessed and found to benefit from the application of the extracts and components of the present invention.

The useful activities are found in one or more fractions derived from mincing and crushing the ginger rhizome. The resulting crush may be dried to generate an active powder form or, alternatively, may be filtered to produce a crush filtrate from which may be generated an "isolate" comprising the components referred to herein as "Zingibain". In any of the foregoing cases - the dried powder, the crush filtrate or the isolate - the preferred active component comprised therein is Zingibain. Zingibain activity may be used consistently and reliably to hydrolyse, in a highly specific manner, a particular target. More particularly,

Zingibain is effective in any situation wherein the target comprises a proteinaceous molecule that comprises a significant percentage of proline residues. The proline residues are preferably preceded by a hydrophilic amino acid. Suitable amino acids include, for example, glutamine, arginine, lysine, asparagine, glutamic acid and aspartic acid. Reference herein to "Zingibain activity" may also be read as "Zingibain activities".

As stated above, the preferred active component comprised within the extract or molecular components thereof is referred to herein as "Zingibain" and it has application in a wide range of related fields. One field of application wherein the Zingibain activity of the present invention finds wide-ranging use is in industries engaged in the preparation of food and feed for humans and animals, respectively. Zingibain may be used to improve the quality characteristics of edible material. Other fields of application extend to and include a means for the maintenance of health of animals, including human animals as well as companion and farm animals, and to prophylaxis and treatment of diseases/disorders of animals including humans. Additional applications encompass use of the molecular components as tools in cell and molecular biology, and industrial applications such as the production of ethanol from cereals and the treatment of waste products.

Particularly preferred edible materials are those which constitute feed and/or food for animals, including human animals. Resulting products are characterized in that they are, for example, more tender, more palatable or less allergenic than their untreated equivalents. Food and feed products or ingredients thereof may have the improved quality characteristic conferred upon them by prior treatment with the extract and/or molecular components of the present invention.

In one embodiment, the tenderness of meat products for human consumption is increased by prior treatment with the extract and/or molecular components of the present invention. Collagen, the major proline-containing protein of meat, is thereby degraded, leading to a consistency having greater tenderness. Further applications of the extracts and molecular components of the present invention relate to their use in degrading collagenous fibres in tissues wherein their presence is undesirable as, for example, in a cosmetic method of

treatment designed to remove or reduce the presence of collagen in a target tissue.

The plant extract of the instant invention is further useful as a medicament or in the manufacture of a medicament for the treatment and, in some cases, prophylaxis of a disease condition of skin such as, for example, burns, insect bites and stings, abrasions, cancer, psoriasis and other inflammatory disorders.

Disorders of the foregoing type typically involve superficial lesions and/or abnormalities that require topical application of a medicament useful in the treatment thereof. The instant invention, however, provides agents that may be formulated as medicaments for systemic administration. Hence, the extract and/or molecular components thereof are also applicable for treatment and, in some cases, prophylaxis of a broader range of ailments extending to atherosclerosis, tumours, inflammatory diseases, prion-caused disease, forms of dementia, blood disorders, viral infection, *etc.*

In related embodiments, the extract and/or molecular components of the present invention may be applied in a method for the prevention and/or treatment of a range of disease states, including a systemic and/or skin disorder such as those recited above.

The extract and components of the present invention exhibit proteolytic activity directed, in particular, at targets that comprise a conformationally exposed proline residue preceded by a hydrophilic amino acid residue. The preferred proteolytic activity is catalyzed by Zingibain which may be used consistently and reliably to hydrolyse such a target. Therefore, in addition to the applications described above, this property makes Zingibain especially useful in circumstances requiring consistent analytical-grade tools such as in research and development laboratories applying, for example, cell and molecular biological approaches to the investigation of biological questions. Such investigative approaches may require, *inter alia*, reproducible and complete removal of cellular material away from tissue culture containers; dissociation of tissue into single cells for harvesting; reliable target-specific protein degradation, and the like.

Furthermore, reliable target-specific protein degradation is one particular property of Zingibain, which is also sought after in industrial applications, such as in the hydrolysis of the gluten and related proteins in cereals to improve the efficiency of the hydrolysis and fermentation of starch to form ethanol, and in the treatment of waste products comprising unwanted proteinaceous material from plant and/or animal sources, wherein the complete dissociation of the waste material is desirable.

A summary of sequence identifiers used throughout the subject specification is provided in Table 1.

**TABLE 1**

***Summary of sequence identifiers***

| SEQUENCE ID NO: | DESCRIPTION |
|---|---|
| 1 | Amino acid sequence of the component, isolatable from the ginger rhizome fraction designated GP-II, and exhibiting cysteine protease activity |
| 2 | Amino acid sequence of the dominant component, isolatable from the ginger rhizome fraction designated GP-I, and exhibiting cysteine protease activity |
| 3 | amino acid sequence of the bovine 28,600 Da proteinaceous infectious protein (prion) |
| 4 | Amino acid sequence of prion repeat unit from chicken |
| 5 | Amino acid sequence of prion repeat unit from bovine |

## BRIEF DESCRIPTION OF THE FIGURES

**Figure 1** is a representation of the structure of Zingibain, showing the four molecules in the crystallographic unit cell in two different orientations with the helical domains represented by cylindrical tube-like shapes and the β-sheet domains represented by flat rectangular shapes. The locations of the saccharide moieties are also indicated (Choi *et al.*, *Biochem. 38:* 11624-11633, 1999).

**Figure 2** is a graphical representation of the Zingibain activity for the hydrolysis of collagen as a function of temperature. Activity of Zingibain is expressed as $A_{520}$ units released per unit time on the y axis. The temperature is indicated on the x axis.

**Figure 3** is a graphical representation showing Meat Standards Australia Trials. A trial of 180 people from three community groups assessed meat quality from 20 100% Brahman cattle from North Queensland with matching muscles treated with Zingibain and untreated (control). "Tend" is a tenderness score averaged over the cattle and the individual people scores given from 0 to 100. "Juice" is a juiciness score; "Oall" is an overall palatability score, each averaged in the same way for as for tenderness. MQ4 is a weighted score over the 4 parameters used by the MSA to give an overall grade to the meat.

**Figure 4** is a pictorial representation showing the trinodular structure of the fibrinogen molecule. (refer to Retzinger, 2000; http://oz.uc.edu/~retzings/fibrin().htm). It is a dimeric plasma protein, with each monomer unit being composed of disulfide linked chains Aα, Bβ and γ. The amino terminal of 16 residues of Aα and 14 residues of Bβ are called fibrinopeptides A (FpA) and B (FpB), respectively. The dimer is a 450 angstrom long "rope" with the amino-terminal chains forming a globular domain (the so-called disulfide or E knot) where 11 disulfide bonds hold the six chains together, and the carboxy-terminal chains for Bβ and γ end in the globular D domains, whereas the carboxy-terminal chains for Aα extend back into the central E domain. Except for the α-C domains, the regions between the globular domains form α-helical coiled-coil structures.

**Figures 5A-C** are graphical representations showing the change in weight over time of animals (dogs) when provided with food supplemented with Zingibain. **(A)** a 2.5-year-old Airedale Terrier bitch whose weight changed from 18.3 kg on Day 0 to 23.3 kg by Day 87; **(B)** a 5-year-old Kerry Blue Terrier dog; by Day 120, his weight had increased by about 4 kg from his minimum; **(C)** the fluctuation in weight of 7-year-old Miniature Schnauzer bitch correlated with the addition and removal of Zingibain supplement from her food. Refer to Example 7 for additional data and information.

## DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

The present invention is predicated in part on the observation that members of the Zingiberaceae plant family comprise an extractable fraction, which fraction or components thereof exhibit properties useful in a range of applications. The preferred species is *Zingiber officinale*, also known as ginger. Other members of the Zingiberaceae plant family are, however, not precluded and are intended to fall within the scope of the present invention. Examples of other species of *Zingiber* include *Z. mioga, Z. cassumunar* and *Z. zerumbet*. Reference hereinafter to a "ginger plant" is not intended to exclude species fo *Zingiber* other than *Z. officinale*.

The extractable fraction of the instant invention is derived from the ginger rhizome. This part of the ginger plant has been used as a spice in food preparation, and as a non-specific "herbal remedy" for a diverse range of conditions. However, until the advent of the present invention, there was no understanding of the underlying basis for any efficacious effect was previously only sometimes sporadically imparted. In the context of the present invention, however, commercial quantities of various extracts and components of the ginger rhizome are able to be made available. The present invention, therefore, identifies, explains and delineates a wide range of useful applications for definable *Z. officinale* extracts and components thereof. The extracts and components thereof are found to comprise *inter alia* hydrolytic activity, capable of acting highly specifically on proteinaceous molecules that comprise a conformationally exposed proline residue preceded by a hydrophilic amino acid residue.

Accordingly, one aspect of the present invention is directed to the use of a rhizome from a species of *Zingiber* species rhizome in the manufacture of an extract or a molecular component thereof, which is capable of hydrolysing proline-containing proteins.

Preferably, the species of *Zingiber* is *Z. officianale*. The present invention, however, extends to all genera and species of family Zingiberaceae.

Accordingly, in a preferred embodiment, the present invention is directed to the use of *Z. officinale* rhizome in the manufacture of an extract or a molecular component thereof, which is capable of hydrolysing proline-containing proteins

Areas of applicability extend from animal and human feed/food and health maintenance, and disease prophylaxis and treatment, to laboratory and industrial applications in the life sciences, including cell and molecular biology applications, and extend further to industrial applications such as the production of ethanol from cereals, and the treatment of waste products.

Another aspect of the present invention, therefore, is directed to the use of *Z. officinale* rhizome in the manufacture of an extract or a molecular component thereof, which is capable of hydrolysing proline-containing proteins, for producing edible materials exhibiting improved quality characteristics.

In a related embodiment, the present invention is directed to the use of an extract of the *Z. officinale* rhizome, wherein said extract comprises molecular components capable of hydrolysing proline-containing proteins, in the manufacture of edible materials exhibiting improved quality characteristics.

Reference to "*Z. officinale*" or "*Zingiber officinale*" or "ginger plant" is to be read as including other species or genera of the Zingiberaceae family which have similar properties.

The "molecular components", which are comprised in and isolatable from an extract of the *Z. officinale* rhizome, are enzymes the function of which is to degrade proteins. These enzymes are generally referred to as proteolytic enzymes or proteases, and they function by hydrolysing peptide bonds within the amino acid sequences that constitute proteins. As will be well known to one skilled in the art, proteases are ubiquitous in nature and are many and varied in their structure and particular preferred substrate. They are, therefore, generally grouped into like kinds, according to their usual target. One group of proteases is

referred to in the art as "cysteine proteases", in which a thiol group of a cysteine residue is the nucleophilic group involved in attacking and hydrolysing a peptide bond. Representative members of the "cysteine protease" group of proteolytic enzymes include, for example, papain, bromelain and ananain, ficin and actinidin. These molecules are isolatable from papaya (*Carica papaya*), pineapple (*Ananas comosus*), figs, and kiwi-fruit (*Actinidin chinensis*), respectively.

The molecular components that provide the useful activity of the present invention are found in a fraction derived from mincing and crushing the rhizome of *Z. officinale*. The terms "*Zingiber officinale*", "*Z. officinale*" and "ginger" may be used interchangeably throughout this specification. A number of different formulations may be derived from processing the minced ginger rhizome. The minced tissue may be dried to generate the spicy ginger known to culinary aficionados. Alternatively, the minced rhizome may be extracted to produce a "ginger crush", the solution of which comprises the desired active molecular components of the present invention.

This ginger crush may be dried to generate an active powder form or, alternatively, may be filtered to produce a crush filtrate from which may be isolated Zingibain, which is regarded herein as one of the molecular components of the ginger plant extract. In any of the foregoing formulations - the dried powder, the crush or its filtrate or the isolate - the preferred activity is due to a Zingibain extract. Reference herein to "molecular components" includes a component or extract having the characteristics of Zingibain.

"Zingibain", as used herein, refers to a protein fraction, isolatable from ginger rhizome, and comprising proteolytic activities of at least one or two or three closely related enzyme fractions, separable by, for example, DEAE-cellulose chromatography. One of the fractions comprises the GP-II proteases. Another fraction, referred to as "GP-I", comprses two highly homologous proteases. All three proteolytic enzymes are comprised in the dried powder, the crush or its filtrate or the isolate as described herein. Hence, reference to "molecular components" is a reference to any one of or, alternatively, all three proteolytic enzymes. Similarly, throughout this specification, a reference to "Zingibain" is to be

understood to be a reference to the unseparated protease fraction comprising all three protease enzyme activities, or to any one or more of the said protease activities.

The protease activities comprised in Zingibain are identified as being additional members of the "cysteine protease" group. More particularly, Zingibain is a proline-specific cysteine protease. Accordingly, Zingibain is effective in any situation wherein the target is a proteinaceous molecule that comprises a significant percentage of proline residues.

In the context of the present invention, "significant percentage" is to be understood as an amount of proline in excess of about 5%, which is higher than normal in proteins and which gives a greater chance of proline being preceded by a hydrophilic amino acid residue in an exposed site for successful hydrolysis. Preferably, the percentage of proline is less than about 60%, more preferably less than about 50%, even more preferably less than about 40%, still more preferably less than about 30%, and most preferably less than about 20%. Hence, a reference herein to a "proline-containing protein" is to be understood to be a reference to a proteinaceous molecule that comprises a significant percentage of proline residues, as hereinbefore defined.

Without intending to limit the present invention to any one theory or mode of action, it is proposed that Zingibain degrades its protein targets by hydrolysing peptide bonds between a proline amino acid residue and a preceding amino acid residue; that is, between proline and the residue which comes immediately before it in the amino acid sequence, reading from the N-terminal. For optimal hydrolytic effect, a proline residue is preferably preceded by a hydrophilic amino acid. Suitable hydrophilic amino acid residues include, for example, glutamine, arginine, lysine, asparagine, glutamic acid and aspartic acid. The term "hydrolysing" as applied to the effect of a proteolytic enzyme on a peptide bond means that the affected peptide bone is broken or destroyed, and the sequence of the hydrolysed protein is thereby severed or cleaved at that point in the chain. An attacked protein may be broken down, through hydrolysis, into two or into many peptide pieces, depending on the extent of suitable bonds for hydrolysis and on the extent of hydrolysis that actually occurs. Hydrolysis, therefore, destroys proteinaceous material and results in its conversion and/or

degradation into smaller cleaved portions of protein, or peptides, and/or, in its most extreme form, into the amino acid constituents thereof. Destroyed, degraded, converted, cleaved, and/or hydrolysed proteinaceous material no longer exhibits its naturally occurring function.

In accordance with the present invention, ginger rhizomes may be processed to generate extracts that comprise the proline-specific cysteine protease, Zingibain, which is capable of destroying and/or degrading proteins *via* hydrolysis at proline residues.

Accordingly, another aspect of the present invention contemplates the use of *Z. officinale* rhizome in the manufacture of an extract, wherein said extract comprises the proline-specific cysteine protease, Zingibain, for producing edible materials exhibiting improved quality characteristics.

In a related embodiment, the present invention contemplates the use of an extract of the *Z. officinale* rhizome, wherein said extract comprises the proline-specific cysteine protease, Zingibain, in the manufacture of edible materials exhibiting improved quality characteristics.

As used herein, the term "extract" extends to and encompasses any formulation, derived from the *Z. officinale* rhizome, in which Zingibain exists and may be used in accordance with the present invention. "Extract" therefore extends to dried, powder, ginger crush, crush filtrate and isolate, as described above, and any other suitable formulation. The terms "extract" and "Zingibain" are used herein interchangeable.

In accordance with the methods and applications of the present invention, ginger extracts may be applied usefully in the manufacture of edible materials that exhibit improved quality characteristics. "Edible materials" includes and encompasses animal- and/or plant-derived matter, which is used in the preparation of any item to be consumed by animals, including human animals, as a nutrient source. Edible materials includes the ingredients that are used in the preparation of manufactured feed and/or food items and extends to and

encompasses the manufactured feed and/or food items so prepared. The term "feed" generally refers to such items when consumed by animals other than humans; correspondingly, the term "food" generally refers to such items when consumed by humans. In this context, "animals" includes both companion animals, such as cats, dogs and horses, and production animals, such as pigs, goats, sheep, chickens, aquatic species and cattle, *inter alia*.

Common edible items are comprised, for example, of grains such as cereals and legumes, and meat as well as meat-derived manufactured products. Any other feed and/or food item, which is consumed as a source of nutrients, falls within the scope of this aspect of the present invention.

Accordingly, this application of the present invention provides for the hydrolysis of proline-rich proteins comprised within edible material, the result of which is the improvement in one or more quality characteristic thereof. "Quality characteristics" generally relate both to more readily quantifiable characteristics such as nutritive value and digestive value, and to more qualitative characteristics such as taste value. . Examples of quantifiable characteristics related to nutritive value and/or digestive value include, *inter alia*, total fat content, extent of fat distribution, presence of allergen-causing ingredients, prion content *etc*. Examples of qualitative characteristics related to taste value include, *inter alia*, juiciness and tenderness.

One particular application in this field relates, therefore, to the processing and tenderizing of any material containing the proline-rich natural protein, collagen. Resulting products are characterized in that they are more tender or, alternatively, more palatable than their untreated equivalents. Products amenable to such processing and improvement are generally meat and/or meat-derived products. Palatability may include measures of, for example, juiciness of meat products. Tenderizing of meat may be achieved through the inclusion, in the animal feed or during the manufacturing process, of the application of the extract and/or components of the present invention. Alternatively, the said extract may be administered in a suitable form to the edible meat material just prior to ingestion and/or

preparation for ingestion such as by cooking. One way in which the desired quality improvement may be achieved is through the addition of Zingibain to sauces, marinades and/or stocks, for example.

In this context, the terms "meat" and "meat-derived" also extend to and encompass the flesh tissue of seafood; in particular, that which comprises edible material.

Further characteristics that may be improved through the application of the extract and/or molecular components of the present invention include fat distribution and content; allergenicity; prion content; and feed conversion.

Allergenicity is generally related to the presence of particular proteins in, for example, grains such as cereals (wheat, oats, barley, rye, sorghum, corn *inter alia*) and legumes (chick pea, soybean, lentil, peanut, *inter alia*), of proline-rich proteins that, upon ingestion, elicit an allergic antigenic response. By way of example, grains comprise a proline-rich storage protein, localised in the endosperm, known as glutelin. Wheat glutelin is called glutenin. Similarly, gliadin is another potentially allergenic endosperm storage protein belonging to the group of molecules, unique to seeds of cereals and other grasses, known as prolamins. These allergens are candidates for hydrolysis by the extracts of the present invention, being rich in proline sites suitable for attack by Zingibain.

Other proline-rich proteins, suitable for attack by Zingibain, are also found in plant pollens and, in particular, in plant pollens that are highly allergenic.

The presence of the recently identified highly infectious protein named "prion", in food and/or feed products, has been a critical and hugely expensive problem in, especially, the beef cattle industry of Europe and, in particular, the United Kingdom. The spread of the prion-caused infectious disease known as bovine spongiform encephalitis (otherwise known as "mad cow disease") throughout that entire country lead to the forced destruction of a high percentage of the industry of that country and to significant economic hardship. Moreover, there was widespread concern about the possibility of its spreading into the

human population, through ingestion of contaminated food products.

Importantly, in the present context, the structure of prion shares some features with collagen, including the presence of a repeat region that contains proline in an amino acid unit that is repeated. In chicken prion, for example, there is a 54-amino-acid region with nine repeat units (**PHNPGY**) in which proline is every third amino acid, thereby forming an extended polyproline II helix, as is also found in collagen. While normal prion protein is protease-sensitive, the infectious conformation resists breakdown with many proteolytic enzymes. Given its proline-rich structure, however, prion presents an ideal target for hydrolytic degradation by Zingibain, which preferentially and specifically destroys proline-rich natural proteins. Prior treatment of meat and meat-derived products, therefore, presents a means of rendering the meat "prion-free" and, hence, safe for consumption.

Fat content and distribution through muscle tissue, and feed conversion, may also be advantageously affected by similar applications of the extract and/or components of the present invention.

Edible material which displays values such as decreased allergenicity and decreased fat content, and reduced or eliminated prion content, are regarded as providing a healthier alternative to an equivalent product which does not exhibit such characteristics. Such "improved quality characteristics" are therefore sought after and edible material, comprising one or more of these characteristics, is generally preferred by consumers, manufacturers and health educators alike. In some instances, feed/food comprising one or more of these characteristics may be regarded as "functional food". As will be obvious from the above, these quality characteristics apply equally to feed for farm and companion animal consumption as to food for human ingestion.

As already mentioned, one particularly proline-rich natural protein is collagen. Collagen is the most abundant protein in humans, accounting for about 25% of all protein, and its structure is largely conserved in the animal kingdom from the most primitive animals to humans. It is expressed in fibroblast cells. It forms the organic mass of tissues such as skin,

tendon, blood vessels, bone, the cornea and vitreous humor of the eye, and basement membranes. In certain circumstances, it may be desirable or critical to remove and/or reduce the amount or presence of collagen from a particular tissue site. Examples include collagen fibres entangled in blood clots and in dead tissue around burn wounds.

Accordingly, a related embodiment of the present invention is directed to the use of *Z. officinale* rhizome in the manufacture of a medicament comprising an extract or a molecular component thereof, which is capable of hydrolysing proline-containing proteins, for the removal or reduction of collagen in a target tissue.

In an alternative embodiment, the present invention is directed to the use of an extract of the *Z. officinale* rhizome, wherein said extract comprises molecular components capable of hydrolysing proline-containing proteins, in a cosmetic method of treatment designed to remove or reduce the presence of collagen in a target tissue.

"Target tissues" include any tissue wherein collagen is present and wherein, for cosmetic or health purposes, the extent or amount thereof should preferably be, to a greater or lesser extent, reduced. Target tissues contemplated herein include those already cited above; namely, skin, tendon, blood vessels, bone, the cornea and vitreous humor of the eye, and basement membranes. Other tissues, however, may also be encompassed within the intended scope of the present invention, provided that the removal and/or reduction of the amount or presence of collagen from a particular tissue site is desirable and/or critical, and that it may be achieved by the hydrolysis with Zingibain.

Without limiting the invention to any one theory or mode of action, it is proposed that the extract or molecular component of *Z. officinale* rhizome specifically hydrolyses proteins that comprise a significant percentage of proline residues. Particularly preferred proline-rich natural proteins include, but are not limited to, collagen, prion, fibrin, fibrinogen, amyloid beta protein precursor, and particular cell membrane proteins including receptors *etc., inter alia*. Since these molecules are involved in many cellular and biochemical processes, the ginger rhizome extract referred to herein as Zingibain has applications, even

more widely, in preventing and/or treating the effects of biochemical processes that may be undesirable and/or deleterious to health. Such processes may be superficial – affecting, for example, skin – or they may be systemic.

The plant extract of the instant invention is therefore useful as a medicament or in the manufacture of a medicament for the treatment and, in some cases, prophylaxis of a disease condition of skin such as, for example, burns, insect bites and stings, abrasions, cancer, psoriasis and other inflammatory disorders.

Accordingly, another aspect of the present invention is directed to the use of an extract of the *Z. officinale* rhizome, wherein said extract comprises molecular components capable of hydrolysing proline-containing proteins, in the preparation of a medicament for the prophylaxis and/or treatment of a skin disorder in a subject.

Reference herein to "prophylaxis" and "treatment" is to be considered in its broadest context. The term "treatment" does not necessarily imply that a subject is treated until total recovery. Similarly, "prophylaxis" does not necessarily mean that the subject will not eventually contract a disease condition. Accordingly, prophylaxis and treatment includes amelioration of the symptoms of a particular disorder or condition, or preventing or otherwise reducing the risk of developing a particular disorder or condition. The term "prophylaxis" may be considered as reducing the severity or the onset of a particular disorder. "Treatment" may also reduce the severity of an existing condition.

In this context, a "subject" may be a human or an animal subject. Skin disorders of the foregoing type, which may be amenable to prophylaxis and/or treatment in this manner, typically involve superficial lesions and/or abnormalities that require topical application of a medicament useful in the treatment thereof. Such disorders include, for example, burns, insect bites and stings and abrasions. However, it should be understood that the present invention is not limited thereto but extends to encompass more serious diseases, such as cancer, psoriasis and other inflammatory disorders.

In this regard, the instant invention provides agents that may be formulated as medicaments for systemic administration. Hence, the extract and/or molecular components thereof are also applicable for treatment and, in some cases, prophylaxis of a broader range of ailments extending to atherosclerosis, tumours, inflammatory diseases, prion-caused disease, dementia, blood disorders, viral infection, *etc*

Accordingly, a further aspect of the present invention contemplates the use of *Z. officinale* rhizome in the manufacture of a medicament comprising an extract or a molecular component thereof, which is capable of hydrolysing proline-containing proteins, for the prophylaxis and/or treatment of a systemic disorder in a subject.

One possible life-threatening event wherein the application of Zingibain may provide effective prophylaxis is the transmission of infectious prion proteins through, for example, blood transfusion and/or tissue transplantation and/or contaminated surgical equipment or blood processing equipment. As mentioned above, while the infectious prion protein conformation resists breakdown with many proteolytic enzymes, its proline-rich structure makes prion an ideal target for destruction by Zingibain. Without wishing to limit this aspect of the invention to any one theory or mode of action, it is proposed that infectious prions may be transmitted to blood and/or transplant recipients or patients undergoing surgery, where they cause disease by inducing normal prion molecules to change conformation to the disease-causing structure. The transmission of Spongiform Encephalopathies in this manner is a major concern as the prion would be very difficult to detect, and the disease takes many years to produce symptoms. Prior treatment of blood/tissue with Zingibain and decontamination of surgical and blood-processing equipment obviates this dangerous possibility.

Other disorders wherein the application of the extract of the present invention may provide efficacious include other blood disorders, atherosclerosis, cancer tumours, whether or not metastatic, dementia, inflammatory diseases, and viral infections. All such disorders are to be understood as being encompassed by the term "systemic".

By way of further example, other particularly preferred proline-rich natural proteins, susceptible to proteolytic degradation by the extracts of the present invention include, in addition to collagen and prion, fibrin and fibrinogen. Fibrin and fibrinogen have extremely important functions in animals and, at the same time, are associated with the occurrence of some of the more common diseases, such as thrombosis, inflammation, cancer, and atherosclerosis. For example, polymerised, cross-linked fibrin forms blood clots causing thrombosis.

These proteins – fibrin and fibrinogen – also have proline residues in key positions, immediately preceded by suitable hydrophilic amino acid residues, and are therefore susceptible to effective hydrolysis by the Zingibain-comprising formulations contemplated herein. Therefore, life-threatening clots may be degraded. Furthermore, by hydrolysing fibrinogen and other members of the blood-clotting cascade, such as prothrombin, Zingibain is also able to prevent the formation of blood clots.

Fibrin and fibrinogen are also closely associated with inflammation. As used herein the term "inflammation" should be interpreted in its broadest sense to indicate a protective response of the body to tissue injury or destruction. Again without wishing to limit the present invention to any one theory or mode of action, it is understood that thrombin and factor XIIIa, which are generated immediately at a site of tissue damage, convert intra- and extravascular fibrinogen at the site to cross-linked fibrin. The fibrin meshwork entraps blood cells, limiting blood loss from the site. It further confines to the site inflammatory cells such as, for example, platelets, granulocytes, monocytes and lymphocytes, which would otherwise circulate. Some of these cells express on their outer surface cellular adhesion molecules that, when activated, have significant affinity for fibrin and/or fibrinogen. It is therefore proposed that, within the inflammation site, fibrin and/or fibrinogen is able to adhere to a variety of cells, thereby keeping them in the location of the inflammation.

As a consequence of their role in inflammation, fibrin and/or fibrinogen are also important in the promotion of tumor growth. Moreover, they have also been implicated in

atherosclerosis, another disease associated with inflammation. Without limiting the application of the invention to any one theory, the atherosclerosis plaque consists of a deposit of extracellular hydrophobic lipids, lipid-laden macrophages, smooth muscle cells and proteins embedded just beneath the endothelial lining of large arteries, including fibrinogen and its degradation products. There is a positive correlation between the fibrin and/or fibrinogen content of the plaque and its lipid content, and plasma fibrinogen level is an independent risk factor for atherosclerotic cardiovascular disease.

Hence, targeted proteolytic degradation of fibrin and/or fibrinogen by the extracts of the present invention may be used to reduce and/or eliminate an inflammatory response, in situations where its occurrence and/or extent is inappropriate, unwanted and/or undesirable. Undesirable effects of fibrin and/or fibrinogen – including blood clotting, inflammation, atherosclerosis and tumour growth – may be prevented, ameliorated or otherwise reduced by the application of the *Z. officinale* rhizome extract and/or components thereof referred to herein as Zingibain.

Virus cell-membrane proteins commonly are proline-rich and have multiple sites for hydrolysis by Zingibain. These proteins are essential for host-cell invasion and other functions, and their cleavage by Zingibain inhibits the viral infection.

Accordingly, yet another aspect of the present invention is directed to a method of treating and/or preventing a skin disease and/or abnormality in a subject, said method comprising contacting said diseased and/or abnormal skin with an effective amount of a medicament comprising an extract of the *Z. officinale* rhizome, wherein said extract comprises molecular components capable of hydrolysing proline-containing proteins, for a time and under conditions sufficient to prevent, ameliorate or otherwise reduce symptoms of said disease and/or abnormality.

In a related embodiment, the present invention contemplates a method of treating and/or preventing a systemic disorder, said method comprising administering to a subject in need thereof an effective amount of a medicament comprising an extract of the *Z. officinale*

rhizome, wherein said extract comprises molecular components capable of hydrolysing proline-containing proteins, for a time and under conditions sufficient to prevent, ameliorate or otherwise reduce symptoms of the disorder.

The active component of the medicament is contemplated to exhibit therapeutic activity when administered in an "effective amount" that depends on the particular case. By "effective amount" is meant an amount necessary to at least partly obtain the desired response, or to delay the onset or inhibit progression or halt altogether the onset or progression of a particular condition being treated. The amount varies depending upon the health and physical condition of the subject being treated, the taxonomic group of the subject being treated, the degree of protection desired, the formulation of the composition, the assessment of the medical situation and other relevant factors. It is expected that the amount will fall in a relatively broad range, which may be determined through routine trials. Considering a human subject, for example, from about 0.1 mg to about 4 mg of active component may be administered per kilogram of body weight per day. Dosage regimes may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily, weekly, monthly or other suitable time intervals or the dose may be proportionally reduced as indicated by the exigencies of the situation.

In accordance with the applications of the present invention, medicaments comprising the extracts and or components disclosed herein may be formulated, for use in conjunction with the instant methods, *via* topical administration or *via* systemic administration, depending on the nature of the subject's disorder. Appropriately formulated medicaments may then be utilised in the treating and/or preventing disease, whether a skin abnormality or disease, or a systemic disorder such as those referred to above. Such medicaments may be administered to a subject in any one of a number of conventional dosage forms and by any one of a number of convenient means. As already mentioned, "subject" may refer to any animal including but not limited to a human.

Contemplated suitable dosage forms of the active component include tablets, troches, pills, capsules, creams and the like, all of which may also contain additional components, as follows: a binder such as gum, acacia, corn starch or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid and the like; a lubricant such as magnesium stearate; a sweetening agent such as sucrose, lactose or saccharin; a flavouring agent such as peppermint, oil of wintergreen or cherry flavouring. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills or capsules may be coated with shellac, sugar or both. A syrup or elixir may contain the active compound, sucrose as a sweetening agent, methyl and propylparabens as preservatives, a dye and flavouring such as cherry or orange flavour. Of course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the active compound(s) may be incorporated into sustained-release preparations and formulations.

Pharmaceutically acceptable carriers and/or diluents include any and all solvents, dispersion media, coatings, anti-bacterial and anti-fungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art and except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic composition is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

The active component may be administered in a convenient manner such as by oral, intravenous (where water-soluble), intra-peritoneal, intramuscular, subcutaneous, intra-dermal or suppository routes, or *via* implanting (e.g. using slow release molecules). Alternatively, the active component may be formulated for administration topically, such as by cream or gel. The active component may be administered in the form of pharmaceutically acceptable non-toxic salts, such as alkali or alkaline earth salts, such as sodium, potassium, magnesium or calcium.

Preferred formulations for topical administration include those in which the active component of the present invention is in admixture with a topical delivery agent such as lipids, liposomes, fatty acids, fatty acid esters, steroids, chelating agents and surfactants. Preferred lipids and liposomes include neutral (*e.g.*: dioleoylphosphatidyl DOPE ethanolamine, dimyristoylphosphatidyl choline DMPC, distearoylphosphatidyl choline), negative (dimyristoylphosphatidyl glycerol DMPG) and cationic (dioleoyltetramethyl-aminopropyl DOTAP and dioleoylphosphatidyl ethanolamine DOTMA).

For topical or other administration, the extract and/or components of the present invention may be encapsulated within liposomes or may form complexes thereto, in particular to cationic liposomes. Alternatively, the extract and/or component may be complexed to lipids, in particular to cationic lipids. Preferred fatty acids and esters, pharmaceutically acceptable salts thereof, and their uses are known, such as are described in U.S. Patent 6,287,860.

The extract and components of the present invention exhibit proteolytic activity directed, in particular, at targets which comprise a significant percentage of proline residues. The proteolytic activity, referred to herein as Zingibain, may be used consistently and reliably to hydrolyse such a target. Therefore, in addition to the applications described above, this property makes Zingibain especially useful in circumstances requiring consistent analytical-grade tools such as in research and development laboratories applying, for example, cell and molecular biological approaches to the investigation of biological questions. Such investigative approaches may require, *inter alia*, reproducible and complete removal of cellular material away from tissue culture containers; dissociation of tissue into single cells for harvesting; or reliable target-specific protein degradation, *etc.*

Accordingly, still another aspect of the present invention contemplates the use of *Z. officinale* rhizome in the manufacture of an extract or a molecular component thereof, which is capable of hydrolysing proline-containing proteins, for tissue dissociation and/or harvesting of dissociated cells.

In a related embodiment, the present invention contemplates the use of *Z. officinale* rhizome in the manufacture of an extract or a molecular component thereof, which is capable of hydrolysing proline-containing proteins, for specific cleavage of an identified target.

Furthermore, reliable target-specific protein degradation is one particular property of Zingibain, which is also sought after in industrial applications, such as in the treatment of waste products comprising unwanted proteinaceous material from plant and/or animal sources, wherein the complete dissociation of the waste material is desirable.

Accordingly, yet a further aspect of the present invention is directed to the use of *Z. officinale* rhizome in the manufacture of an extract or a molecular component thereof, which is capable of hydrolysing proline-containing proteins, for degradative treatment of industrial waste products.

Examples of waste products which, in particular, may be amenable to degradation by the methods of the present invention include, *inter alia*, wastes from the meat and seafood processing and other food industries.

The process and cost efficiencies of ethanol production from cereals such as wheat and corn are adversely affected by proteins such as the glutelins and prolamins. The specific cleavage of these proteins by Zingibain simplifies the process of starch hydrolysis and fermentation, and adds value to the Distillers Grains and Solubles (DGS) co-product as a livestock feed.

The present invention is further described by the following non-limiting Examples.

## EXAMPLE 1

### *Zingibain protein fraction*

A protein fraction is extractable from ginger rhizome with phosphate pH6 buffer (Thompson *et al., J. Food Sci. 38:* 652-655; 1973; Ichikawa *et al., J. Jpn. Soc. Food Nutr. 26:* 377-383; 1973; Ohtsuki *et al., Biochim. Biophys. Acta 1243:* 181-184; 1995;). This fraction contains three closely related proteolytic enzymes, that may be separated using DEAE-cellulose chromatography into two bands, GP-I containing two enzymes and GP-II containing one enzyme (Ichikawa *et al.*, 1973, *supra*). These three enzymes, each with a molecular weight of about 29,000 Da, can be precipitated from the extract with acetone or ethanol leaving some small contamination from two proteins with molecular weights of 14,000 and 10,000 Da. The dominant component of GP-I has 82% homology with GP-II, and the key amino acids for proline specificity are conserved.

The sequence and structure of GP-II have been determined (Choi *et al.*, 1999, *supra*; Choi and Laursen, *Eur. J. Biochem. 267:* 1516-1526, 2000). The enzyme has 221 amino acids, with the chain folded into two domains of about the same size and a cleft separating the two domains. The amino acid sequence of GP-II is set forth in SEQ ID NO:1. Domain I includes residues 13-112 and 215-218, and is mainly α-helical. Domain II includes residues 3-12 and 113-214 and has an anti-parallel β-sheet structure. This overall structure is very similar to other plant cysteine proteases such as papain and actinidin. The protein is 8% glycosylated by weight with two N-linked oligosaccharides at Asn99 and Asn156. Three disulfide bonds stabilize the GP-II protein fold. These are located between Cys24 and Cys65, Cys58 and Cys98, and Cys155 and Cys206. These residues are strictly conserved throughout the papain family. Polar residues are concentrated on the bottom of the molecule, and there is a neutral face with a radius of about 10 angstroms opposite around the active site cysteine. The active site lies in a 5.5 angstrom deep and 9.5 angstrom long cleft at the interface of the two domains. A representation of the structure of GP-II is shown in Figure 1.

The presence of 14,000 and 10,000 Da protein contaminants in Zingibain can be explained by the self-cleavage of GP-II at Q130-P131, giving two fragments with 130 amino acids and 91 amino acids.

Repeated isolations give a consistent product with an activity in excess of 300 U/mg (Dionysius *et al., J. Food Sci. 58:* 780-784; 1993). The enzyme has also been called "proline-specific cysteine protease" (Choi *et al.*, 1999, *supra*; Choi and Laursen, 2000, *supra*). It belongs to the Papain-like family of cysteine proteases, in which the thiol group of a cysteine is the nucleophilic group for attacking and hydrolyzing a peptide bond. This family includes enzymes such as papain from papaya (*Carica papaya*), bromelain from pineapple (*Ananas comosus*), ananain from pineapple, ficin from figs and actinidin from kiwi fruit (*Actinidia chinensis*).

The sequence of the dominant component of GP-I has also been determined. This amino acid sequence is set forth in SEQ ID NO:2.

The term "Zingibain", as used herein, includes the unseparated protease-containing fraction as well as isolated and purified sub-components thereof.

## EXAMPLE 2

### *Hydrolysis of collagen by Zingibain*

Collagen is the most abundant protein in humans, accounting for about 25% of protein, and its structure is largely conserved in the animal kingdom from the most primitive animals to humans. It is expressed in fibroblast cells. It forms the organic mass of skin, tendon, blood vessels, bone, the cornea, vitreous humor of the eye, and basement membranes. It polymerizes into a triple-stranded helix with each stand over 1,000 amino acids. The major form of collagen in most species is designated as collagen I and has two $\alpha 1(I)$ chains and one $\alpha 2$ chain, $[\alpha 1(I)]_2\alpha 2$. Cartilage collagen has the structure $[\alpha 1(II)]_3$, and collagen that occurs in various tissues, especially embryo tissue, has the structure $[\alpha 1(III)]_3$. Collagen is very rich in proline and hydroxyproline. The helical region has eleven Glu-Pro, two Lys-

Pro, five Arg-Pro, one Asn-Pro, eleven Ser-Pro and two Gln-Pro sites suitable for hydrolysis by Zingibain.

An azocollagen (azocoll) assay was used to study the hydrolysis of collagen with Zingibain. The azocollagen (Sigma Aldrich) substrate suspension was prepared by mixing 0.1 g washed and ground azocollagen powder with 10 mL of assay buffer (0.1 M sodium phosphate pH 6.0 containing 1 mM DTT and 1 mM EDTA) in a small measuring cylinder on a magnetic stirrer at room temperature. After 30 min, 1 mL of the suspension was transferred with a wide bore micropipette (diameter 2.5 mm) to a glass test tube (150 mm x 13 mm) without depositing any of the suspension on the walls of the tube. The tube was equilibrated for 5 min at the designated temperature in a shaking water bath having a horizontal displacement of 4 cm at a speed of 112 passes per min. The enzyme sample (50 μL) was incubated with the substrate for 30 min with constant shaking in the water bath. The reaction was stopped with 1 mL cold 10% v/v trichloroacetic acid (TCA) and the reaction mixture transferred to a 2 mL microfuge tube. After centrifugation at 12,000 rpm for 5 min, the supernatant was removed and its absorbance read at 520 nm. A sample blank was prepared by incubating 1 mL substrate for 30 min, adding 1 mL 10% f/v TCA and then 50 μL enzyme sample.

The studies showed that the reaction did not conform to Michaelis-Menten kinetics. The absorbance increased with substrate concentration linearly up to the maximum achievable concentration of 5%. The effect of temperature showed an increase in rate above 50°C with a maximum rate at 60°C about four times the rate at 37°C, and with the rate falling off sharply so that at 70°C the rate was reduced to one-fifth the maximum rate. The increase in absorbance at 60°C was found to be linear with time up to 90 min. The reaction rate was relatively linear with enzyme concentration over the range of 25-500 μg Zingibain. These results are set forth in Figure 2.

An SDS-gel electrophoresis study of the hydrolysis of beef-muscle collagen showed that Zingibain was effective at breaking down collagen up to 70°C. The fragmentation pattern differed from that for ficin and papain, and from that for *Clostridium histolyticum*

collagenase which produced large fragments of collagen over 3.5 days whereas Zingibain fully degraded the collagen to low molecular weight fragments of the order of 2,000 Da and less. The study showed Zingibain attacked the helical section of collagen.

Hence, treatment of meat for feed/food with Zingibain rapidly yields fully degraded collagen, making such treatment ideal for use in tenderizing meat for animal consumption.

Another SDS-electrophoresis study of acid-soluble Type I collagen showed that the triple-stranded γ-form of collagen was attacked at low Zingibain levels forming a band not far below the γ-band, with lines also appearing below the bands for the single-stranded $\alpha_1$ and $\alpha_2$ collagen, and with bands for the double-stranded β form and its degradation products. As the concentration of Zingibain was increased, the γ- and β-bands quickly disappeared with only weak α-bands still visible with clear sharp bands of degradation products at lower molecular weights than the 100,000 Da α-form. At higher concentrations of Zingibain, only bands due to Zingibain and low molecular weight fragment products were visible. These observations also support the proposition that Zingibain attacks the helical regions and not simply at the telopeptides at either end of the molecule, as these sections are too small to account for the fragmentation patterns observed.

## EXAMPLE 3

### *Hydrolysis of prion proteins by Zingibain*

Proteinaceous infectious agents, known as "prions", have been identified and characterized over the past two decades. These infectious agents are known to be the causative agent in the spongiform encephalopathies, such as:

- Bovine Spongiform Encephalitis (BSE), or Mad Cow Disease;
- Scrapie, the disease of sheep and goats;
- Creutzfeldt-Jakob Disease of humans, of which 10-15% of cases are due to heritability while some cases are caused inadvertently through operation infection, and perhaps through blood transfusions;

- Gerstmann-Straussler-Scheinker Disease; and
- Fatal Familial Insomnia.

They consist mainly, if not exclusively, of a protein called prion protein (designated PrP). It is known that one form of PrP causes the disease (PrPsc), and a second form (PrPc - the normal form) does not.

***(a) Prion structure***

The difference is apparently caused by a conformational change in the protein structure. Normal PrPc consists primarily of α-helices, and the diseased PrPsc consists primarily of β-sheets. Apparently, the presence of the PrPsc can cause the normal PrPc to change conformation and become the infectious PrPsc. It is further thought that, for humans or other animals carrying a mutated gene, the mutation may render the PrPc susceptible to flip from the α-helix to the β-sheet conformation. This change takes time to occur, as does the accumulation of enough infectious PrPsc to damage the brain sufficiently to cause symptoms.

***(b) Proline prevalence and Zingibain susceptibility***

The structure of prions shares some similar features to collagen, including the presence of a repeat region that contains proline in an amino acid unit that is repeated. In chicken prion, for example, there is a 54-amino-acid region with nine repeat units (PHNPGY) [SEQ ID NO:4] in which proline is every third amino acid, thereby forming an extended polyproline II helix (refer to Figure 3), as is also found in collagen.

Normal PrP is protease-sensitive. However, PrPsc in infected brains resists breakdown with proteases. Given the structure of prions, however, they represent ideal targets for hydrolytic degradation by Zingibain. Zingibain, having proline-rich natural proteins as its preferred target molecule, quickly renders PrPsc harmless through proteolytic cleavage.

Bovine prion is a 28,600 Da protein, having the sequence seen below (also set forth in SEQ ID NO:3):

| | | | |
|---|---|---|---|
| 1 | MVKSHIGSWI | LVLFVAMWSD | VGLCKKRPKP |
| 31 | GGGWNTGGSR | PGQGSPGGN | RYPPQGGGG |
| 60 | WGQPHGGG* | WGQPHGGG* | WGQPHGGG* |
| 84 | WGQPHGGG* | WGQPHGGG* | |
| 100 | GWGQGGTHGQ | WNKPSKPKTN | MKHVAGAAAA |
| 130 | GAVVGGLGGY | MLGSAMSRPL | IHFGSDYEDR |
| 160 | YYRENMHRYP | NQVYYRPVDQ | YSNQNNFVHD |
| 190 | CVNITVKEHT | VTTTTKGENF | TETDIKMMER |
| 220 | VVEQMCITQY | QRESQAYYQR | GASVILFSSP |
| 250 | PVILLISFLI | FLIVG | |

The 18 prolines are indicated in bold face "**P**". Of these, 16 have a hydrophilic residue at $P_1$ (the position immediately prior to the "P"). The 5 prolines in the repeat units "WGQPHGGG" [SEQ ID NO:4], indicated with an asterisk, thus: *, have glutamine at $P_1$; Pro28, Pro148, and Pro166 have arginine; Pro30, Pro113, and Pro116 have lysine; Pro42, Pro53 and Pro169 have tyrosine; and Pro47 and Pro249 have serine.

All of these prolines, if exposed to attack by Zingibain, are susceptible to hydrolysis. Moreover, the prions are universally structured for multi-site hydrolysis by Zingibain, exactly as is collagen. As the more exposed regions are cleaved, the internal structure is exposed for hydrolysis, so that the protein is completely destroyed.

### *(c) Prevention of prion transmission through blood transfusion*

One possible life-threatening event that is prevented through the application of Zingibain is the unexpected/undetectable transmission of infectious prion protein through, for example, blood transfusion. PrP can be transmitted to blood recipients and cause disease by interacting with PrPc molecules, inducing them to change conformation to the disease-causing β-sheet-prevalent PrPsc structure. The possible transmission of Spongiform Encephalopathies through blood transfusion is a major concern because the prion would be very difficult to detect, and the disease takes many years to produce symptoms. Prior treatment of blood to be transfused with Zingibain obviates this dangerous possibility.

Similarly, surgical equipment and blood-processing equipment may be decontaminated if exposed to prion molecules, preventing the transmission of the disease.

***(d) Preparation of prion-free food and feed***

Meat and meat products, prepared for both the human food and animal feed markets, may be routinely treated with Zingibain to cause the degradation of potentially-present prions, rendering the to-be-consumed product prion-free and hence safe.

## EXAMPLE 4

***Zingibain-eradication of allergenicity***

Many commercially important plant proteins are proline rich. Even the pollens collected by bees from Australian native trees are particularly rich in proline: the amino acid Pollen Analysis for eight common pollen sources in 1990/91 gives the following mean values, in percentage (Stace, "Protein Content and Amino Acid Profiles of Honeybee-Collected Pollens" Bees'N'Trees Consultants, Lismore, NSW, Australia, 1996, 2480):

| | | | | | |
|---|---|---|---|---|---|
| Threonine | 3.51 | Leucine | 6.25 | Lysine | 5.90 |
| Valine | 4.70 | Isoleucine | 3.83 | Histidine | 2.13 |
| Methionine | 1.75 | Phenylalanine | 3.75 | Arginine | 5.3 |
| Tryptophan | 2.65 | Aspartic acid | 8.62 | Serine | 4.43 |
| Glutamate | 10.36 | **Proline** | **11.69** | Glycine | 4.23 |
| Alanine | 5.00 | Cystine | 0.84 | Crude protein | 22.7% |

In instances where these pollens are allergenic, Zingibain removes the allergens through protein hydrolysis.

Another particular example involves cereals. Cereals are important protein sources and are processed into bread, cakes, pasta, pizza bases, noodles, breakfast cereals, fermented drinks, animal feeds, as well as having novel applications in food and non-food industries.

Wheat, for example, comprises a proline-rich glutelin, called glutenin. Glutelins are storage proteins located in the endosperm. They are rich in asparagine, glutamine, arginine and proline, and are low in lysine, tryptophan and methionine (Abrol *et al., Aust. J. Agric. Res. 22:* 197-202, 1971; Derbyshire *et al.*, D. *Phytochemistry 15:* 3-24; 1976; *supra*; Kirkman *et al., J. Sci. Food Agric. 33:* 115-127; 1982; Larkins, B.A. "Seed storage proteins: characterization and biosynthesis" in "The Biochemistry of Plants" Stumpf, P.K.; Conn, E.E. (eds) Academic Press NY, Vol 6, pp449-489). Wheat's glutenin is a large polymer with a molecular weight greater than a million. When the disulfide binds are reduced, two fragments are isolated, a high molecular weight sub-unit with a molecular weight of 80-160 kDa, and a low molecular weight sub-unit similar to α-gliadin. Gliadin is another potentially allergenic endosperm storage protein belonging to the group of molecules, unique to seeds of cereals and other grasses, known as prolamins. Wheat α-gliadin has five domains, the first of which comprises a non-repeat N-terminus sequence plus a repeat sequence rich in glutamine, proline and aromatic amino acids.

All allergenic epitopes that have been identified for glutenin and the gliadins have at least one proline in the epitope sequence preceded by a hydrophilic amino acid residue (Vader *et al., Gastroenterology 122:* 1729-1737; 2002). Hydrolysis with Zingibain degrades these sequences, concomitantly removing the allergenic effect of the intact protein. Wheat flour is thereby relieved of its allergenicity.

## EXAMPLE 5

### *Removal of gluten response in person with Celiac Disease*

A person with a well established history of symptoms of celiac disease, who had been on a strict, long-term "gluten-free" diet was provided with a daily intake of various products made from wheat flour that included, in the ingredients, the filtered ginger crush product.

The products included buttercake, devilishly dark chocolate torte, French bread and egg pasta (spaghetti). They were prepared as follows:

*(a) Buttercake*

An oven was pre-heated to 180°C. A cake tin was brushed with melted margarine, and the base lined with baking paper. Using an electric beater, 125 g margarine and ¾ cup castor sugar were beaten in a small mixing bowl until light and creamy. Two eggs, lightly beaten, were added gradually, beating well after each addition. One teaspoon of vanilla essence was added, and the mixture beaten well until combined.

The mixture was transferred to a large bowl. Using a metal spoon, 2 cups of sifted self-raising flour were folded in, alternatively, with ½ cup milk. The mixture was stirred until just combined, and 1 teaspoon of filtered ginger crush solution was added and the mixture again stirred until almost smooth.

The mixture was spooned into the prepared tin, and the cake baked for 45 mins, when a skewer inserted into the centre of the cake came out clean. The cake was left in the tin for 10 mins, and then turned onto a wire rack to cool.

*(b) Devilishly dark chocolate torte*

The base and sides of a deep 23 cm square cake pan were greased with margarine and the base of the pan was covered with baking paper.

Margarine (185 g) was melted in a saucepan, and removed from the heat. One cup of double-strength short-black coffee was stirred in, combined with 150 g chopped dark chocolate and ½ cup castor sugar, and the mixture stirred until smooth. The mixture was placed in a large bowl of an electric mixer. To this was beaten-in, in three batches, a sifted mixture of 1 cup self-raising flour, ¾ cup plain flour, and 2 tablespoons cocoa, followed by 2 eggs, 1 teaspoon vanilla essence and 1 teaspoon filtered ginger crush Zingibain solution.

The mixture was poured into the prepared pan, and baked in a slow oven (150°C) until firm (1.25 hr). The cake was stood for 5 mins before being turned onto a wire rack to cool.

The cake was cut in half, and each half split into three layers. A layer of cake was placed on a serving plate and spread thinly with raspberry jam. A thin layer of a filling was then made by combining 200 g hot melted dark chocolate and 125 g margarine in a bowl, stirring in ¼ cup sifted pure icing sugar, cooling to room temperature and beating with a wooden spoon until the filling was thick and spreadable. This was topped with another layer of cake, which was sprinkled with a little Crème de Cacao, then spread thinly with the filling. The layering was repeated with the remaining cake, liqueur and filling. The layered cake was refrigerated for several hours until it was firm.

Filling ($^2/_3$ cup), which had been reserved, was spread evenly over the cake.

*(c) French bread*

A Breville Master Excel Bread and Dough Maker was used with its recipe (except for the added filtered ginger crush Zingibain solution) for French Bread (750 g loaf), with the following ingredients added in the set order: 310 ml water; 1.5 teaspoons filtered ginger crush Zingibain solution; 2 teaspoons extra virgin olive oil; 1.5 teaspoons salt; 2 teaspoons sugar; 3 cups (450 g) unbleached plain flour (12% protein); 1 teaspoon Bread Improver; and 1.5 teaspoons dry yeast.

A medium setting was used, which had the following program: 1st knead; 2nd knead; 1st rise; punch down; 2nd rise; punch down; 3rd rise; bake for a total time of 3.36 hr. The bread rose to close to the top of the container.

*(b) Egg pasta (spaghetti)*

A Breville Master Excel Bread and Dough Maker was used with its recipe (except for the added filtered ginger crush Zingibain solution) for Egg Pasta Dough, with the following

ingredients added to the bread pan in the set order: four lightly beaten eggs (60 g); 1.5 teaspoons filtered ginger crush Zingibain solution; 1 tablespoon extra virgin olive oil; 1 teaspoon salt; 2 cups (300 g) plain flour; 1 cup (170 g) semolina.

A pasta dough setting of "8" was used with a processing time of 13 minutes. The dough was rolled into a cylinder using a buckwheat "gluten-free" flour dusting on the plastic sheet. It was cut into portions and put through a pasta maker to prepare spaghetti, which was allowed to dry for 1 hr prior to packaging.

In week 1, the person ate one slice of the buttercake each afternoon. In week 2, the person ate one slice of the chocolate cake each afternoon. In week 3, the person ate two slices of the bread for lunch each day, and on the third day ate a dish of the spaghetti for dinner.

The person monitored closely any response of her body to the food. There was no sign of any adverse effect. Considering that her normal adverse response time, after eating a product with wheat flour, was about 2 hr before diarrhoea and flatulence set in, the lack of any sign of response, after consuming the above range of wheat flour products, was seen as evidence supporting the conclusion that the allergenic epitopes of gliadin and glutenin had been removed by the ginger crush Zingibain solution.

## EXAMPLE 6

### *Improved quality of food for human consumption*

The effects of Zingibain on wheat, corn and oats have been examined in terms of the quality of food consumed by humans. The effects were dramatic, as reported by standard "taste test" panels of experts.

#### *(a) Coffee buns*

Coffee buns were made from wheat flour mixed with low-fat milk, one batch containing Zingibain and the other without. The mixture was left overnight at about 37°C. The two

doughs were significantly different: the Zingibain-treated dough was more like a pliable plastic. When mixed with margarine, sugar, eggs, sodium bicarbonate and egg and cooked at 200°C for 15 minutes, the buns were both pleasant to eat. However, in a blind taste-test with experienced cooks, the Zingibain-treated buns were selected as being light, having a good front-palate and a silken after-palate, and having no hint of a "soda-flour" taste that is characteristic of "scones" and as was found for the other buns.

***(b) Bread***

Bread made from wheat flour, which had been sifted with Zingibain and included in a standard milk bread mix, yielded a product that was selected by the taste panel as far superior to the bread made without the Zingibain, with a structure that was much finer and more uniform. One of the panel, who had been trained in bread-making, reported that the structure was more like "gluten-free bread".

***(c) Crepes***

Cornmeal crepes were made from "Mellow Yellow" polenta in a standard recipe with Zingibain sifted with the wheat flour ingredient for one batch, and the flour sifted without Zingibain for the second batch. The two batches of batter were left at 37°C for one hour prior to cooking. A trained cook prepared the crepes ("blind") and reported during the cooking that the two batters were very different, with one staying on the crepe pan exceptionally well and the other displaying characteristics of a typical cornmeal crepe batter, which is always difficult to keep on the pan. Again, when the two batches of crepes were tasted 'blind', the difference was very marked. One batch was reported to be very smooth and uniform with no grittiness, whereas the other was typical of cornmeal crepes with grittiness and inconsistent structure. The panel commented that it was the first time they had eaten a cornmeal crepe that they had enjoyed.

*(d) Uncle Toby's*

Oats were cooked as porridge in the standard way, with the exception that cool tap water was added initially, instead of boiling water. One batch contained Zingibain. In contrast to the batch with no Zingibain, the Zingibain porridge had no firm grains: all the grains had become gelatinous.

*(e) Gluten-free products of Example 5*

The buttercake, devilishly dark chocolate torte, French bread and egg pasta (spaghetti) food items, prepared in accordance with the experiment set forth in Example 4, above, were also classified as extremely palatable by the person undergoing the trial.

These examples show that the addition of Zingibain to food items during prepared results in a more palatable and preferred product.

**EXAMPLE 7**

***Improved quality of feed for animal consumption***

Companion and commercial animal feeds contain proteins from a broad range of sources such as cereals, soy, cottonseed meal, and animal by-products. Enzymes in animal feeds improve the nutritive value of foodstuffs and reduce pollution as a consequence of better utilization of feed. All animals use digestive enzymes that are produced by the animals themselves or by the micro-flora of the gastrointestinal tract, but the feed-conversion efficiency is not 100%. For some animal/feed combinations, up to 25% of the feed is not digested.

Exogenous enzymes are therefore used to break down anti-nutritional factors such as lectins and trypsin inhibitors and allergenic epitopes that are present in many feed ingredients and that are not broken down by endogenous enzymes. These can otherwise interfere with normal digestion, causing poor performance and digestive upsets.

Exogenous enzymes also increase the availability of carbohydrates, proteins and minerals, which are either enclosed within particularly resistant cell walls, and therefore not as accessible to the endogenous enzymes, or are bound up in a form that the animal cannot digest. They also break down specific chemical bonds in raw materials, which that are not usually broken down by the endogenous enzymes, thereby releasing more nutrients.

Young animals, in particular, benefit from exogenous enzymes because of the immaturity of their own digestive system. ("The current feed enzyme market and likely trends" in "Enzymes in Farm Animal Nutrition" Bedford, M.R.; Partridge, G.G. (eds) CABI Publishing Marlborough UK, 2001). Furthermore, the laying-down of meat muscle cells at the time of weaning makes it imperative to provide the weaner with pre-digested protein-rich feed. The normal raw materials for this are skimmed milk powder, whey powder and derivatives, blood and blood plasma products, processed fishmeal, "low antigen" soy proteins, and cooked cereals.

Although the main storage proteins of soybeans, glycinin and β-conglycinin, are implicated in changes to the intestine of young pigs fed with this legume (Li *et al., J. Animal Sci. 69:* 4062; 1992), it will continue to be included in feeds because of its high protein level and low cost. The antigenicity of these proteins is removed by cleaving the antigen epitope, through pre-digesting the feed with Zingibain.

The trypsin inhibitors of soy, unless destroyed before feeding, will cause the pancreas to produce protein-rich secretions with concomitant loss of cells lining the gut (Partridge, , "Considerations in the Formulation of Piglet Creep and Starter Feed" Technical Bulletin, American Soybean Association, 1997). Prior incubation with Zingibain also causes reduction of trypsin inhibitors, through hydrolysis.

A solution of Zingibain is mixed with the protein source at room temperature or at temperatures up to 65°C to pre-digest the feed protein before it is added to other ingredients and pelletized. The latter process sometimes occurs at a higher temperature, where Zingibain is then deactivated. Alternatively, Zingibain is added to the dry feed either

in its active state as a dry powder, or as a solution, just prior to being fed to animals. In addition to improving feed efficiency, Zingibain improves overall animal health.

**EXAMPLE 8**

***Trials of Zingibain in dog food***

The following trials were conducted using Nutrience "Super Premium Adult Active" dog food, manufactured by Rolf C Hagen Inc of Montreal, Canada. It was chosen for the trials following a series of trials of different international "Super Premium" dog foods (with no Zingibain added) at a major boarding kennel in Brisbane.

The following results show that, although it performed better than the other foods, it did not have the healing power or Metabolisable Energy as the food plus Zingibain powder. Nutrience Adult Active contains an undisclosed amount of ginger powder, as well as other herbs and spices, but they had not been prepared by the inventors' proprietary method, which ensures that all beneficial components are at their natural levels and in an active state. The crude protein is guaranteed at 30%, derived mainly from corn, chicken meal and dried egg product.

Dogs were housed in 24 square metre secure, concreted individual enclosures with a total of 18 square metres under cover and with a 6 square metre insulated internal night kennel. The staff feeding and monitoring the dogs did not know when Zingibain was introduced into the feed, or when the level of Zingibain was changed.

***(a) Trial 1***

The aim of this trial was to reduce arthritic pain from a major bilateral hip joint abnormality, and to increase weight to an appropriate level for the breed, age and gender, taking into account the hip problem.

The dog tested is a 2.5-year-old Airedale Terrier bitch, who was about 7 kg under standard weight (weight: 18.3 kg; standard weight: 25 kg), even though she was on a higher than normal daily feed intake (400 g Nutrience Adult Active; normal for 20-30 kg active dog is 260-360 g). Because of inflammation of the hip joints, The dog was finding it increasingly difficult to get up in the morning and to be her normal active self.

Zingibain powder (32 mg) was mixed daily, with the 400 g Nutrience Adult Active dry feed, starting on Day 1. For the period Day 33 to Day 40, only 16 mg Zingibain was added, to see if a lower level had a lesser effect. The dog was monitored regularly in order to determine any effects of the Zingibain on the dog's health and behaviour. Her faeces were collected and weighed wet daily, and each week's collection was air dried separately to determine any changes to Digestibility Percentage. The dog was weighed and inspected by a veterinarian on Day 0 and then from time to time during the trial.

The dog rapidly showed behavioural changes with no obvious signs of arthritic pain, although the looseness in the hip joints was obvious when she ran. She became much more active in her general behaviour, with no reluctance to get up in the morning. The dog's weight changed as shown in Figure 5A, from 18.3 kg on Day 0 to 23.3 kg by Day 87. The reduction in the level of Zingibain from Day 33 to Day 40 affected the weight gain, and when a bitch in an adjacent kennel came into season on about Day 57, and the dog came into season on about Day 78, this also impacted on the weight gain. Her Digestibility Percentage (100[weight feed - weight dry faeces]/weight feed) increased from 79% to about 82% over the 87 days. The dog's average daily weight gain of 57.5 g from her 400 g feed greatly exceeded the 12 g per day expected from the decrease in faecal weight.

Therefore, Zingibain supplementation to feed is able to affect the general health of animals, by reducing arthritic pain from severe joint abnormalities. It is further able to increase the feed's Metabolisable Energy significantly, to allow an animal to gain weight even though the same feed and level of feed (higher than normal) had been eaten for 12 months without a significant change in the animal's weight.

*(b) Trial 2*

In this trial, the aim was to stop major, rapid weight loss and bleeding from the anus, and to restore the health and weight of the dog. The bleeding was thought to result from either multiple gastrointestinal ulcers or cancer, with a possible secondary tumour in the liver causing the severe weight loss.

The dog tested is a 5-year-old Kerry Blue Terrier dog whose normal weight had been about 17.5 kg, which is close to the standard for the breed, age and gender. However, although he continued to eat his 200 g Nutrience Adult Active feed daily, as he had for the previous 12 months, he suddenly lost weight (about 7 kg) over a few weeks and blood was noted in his faeces and, later, severe anal bleeding was observed.

Zingibain powder (16 mg) was added daily to 200 g Nutrience Adult Active dry feed from Day 4 to Day 21. The level of Zingibain was increased to 32 mg from day 22 to day 39; it was reduced to 16 mg for Day 40 to Day 47, when it was increased again to 32 mg per day to see if there was any dependence on the Zingibain level.

From Day 0, the dog's faeces were inspected for any sign of blood, and its anus was wiped with a tissue to look for blood. From Day 8, the faeces were collected daily and weighed, and each 7 day collection was held separately and air dried. The dog's general health and behaviour were monitored closely each day, and he was weighed and inspected by a veterinarian from time to time during the trial.

Within 24 hr of adding Zingibain to the diet (on Day 5), bleeding ceased. No evidence of blood was observed in the faeces or on the tissue and no evidence of bleeding has been found since. The dog continued to eat his whole diet each day, and he showed good health with plenty of energy. His weight loss slowed down and his weight bottomed at 9.6 kg on Day 35, and commenced increasing on Day 64 with a dip at about Day 87 when the bitch in the next enclosure came into season. By Day 120, the dog's weight had increased by

about 4 kg from his minimum (refer to Figure 5B). The dry faecal weight showed no significant trend.

In this instance, Zingibain supplementation to dog food assisted in curing gastrointestinal disease that causes severe blood and weight loss.

*(c) Trial 3*

The aim of this trial was to provide a dog's Maintenance Energy Requirement by administration of 75% of the normal feed, to which was added a Zingibain supplement.

The test dog is a 7-year-old Miniature Schnauzer bitch. She was in excellent condition, at 6.5 kg (standard weight is 6.0-6.5 Kg). She had been on Nutrience Adult Active feed for over 12 months, at 100 g per day. For the duration of the trial, the dog was fed 75 g Nutrience Adult Active dry feed (75% normal feed). On Day 4, Zingibain powder was mixed with the feed: Zena received 8 mg for Day 4 to Day 32, 4 mg from Day 33 to Day 40, 8 mg from Day 41 to 57, 0 mg from Day 58. She was returned to her normal diets on Day 64, when her condition started to be affected by the reduced diet with no Zingibain.

The dog was monitored regularly; her faeces were weighed as above, and she was weighed and inspected by a veterinarian from time to time, as were the other animals in the trials.

For the first three days, when the feed level was reduced without Zingibain, the dog lost weight. However, no further weight was lost when the Zingibain was added, and the weight actually started to increase. When the level of Zingibain was reduced to a half, she lost weight again, but recovered her weight when the level of Zingibain was again increased. When the Zingibain was removed from the feed, the dog showed a loss of condition and a weight loss to the extent that it was deemed necessary to terminate the trial after Day 63 because of the loss of general condition (refer to Figure 5C).

A similar set of results was obtained for another dog.

It is, therefore, apparent that significantly lower levels of feed can be used to achieve the Maintenance Energy Requirement of a dog, if about 1 mg Zingibain per kg dog weight is added to the feed.

## EXAMPLE 9

### *Trials of Zingibain in chicken feed*

In this experiment, the aim was to determine the effect of treating commercial chicken feeds with a solution of Zingibain (1 mg Zingibain per kg chicken, in the average food eaten per day) on the live weight gained and on a number of other parameters as follows: carcase weight, breast meat yield, total protein, fat and ash, and palatability of the meat.

Newly hatched chickens were purchased for the trial. Most were initially a black/grey colour, some with yellow dots. Others were yellow, some with black dots, and there was one brown chicken. They were sufficiently different in their colour patterns so that identification during the trial was possible. Chickens of the same general colour were put in boxes and were divided by 'blind selection' into two indoor pens 1 m x 2.5 m, one for control and one for treated chickens, each with an adequate water supply and four feeding trays. The single brown chicken was put into the "red" pen, which was the pen for the Zingibain treatment. Each chicken was weighed at about 11:30 a.m. on (Day 1), about 2 hours after hatching. Subsequently, chickens were weighed each day at about 7:00 a.m. and 5:00 p.m., and after day 20, the weight of feed remaining uneaten in each pen was weighed twice a day at these times. Each pen had a 60-watt bulb light set at an appropriate distance from the floor to ensure the chickens were kept warm. The chickens were free to eat 24 hr each day (*ad libitum*) from four feed trays.

Commercial feeds used in the trial were purchased from local produce agents. The feeds ranged in protein content from 14 to 20%. The feeds were treated with water, and Zingibain was added to the trial feed to give 2 mg Zingibain per kg chicken per day.

The chickens were coded and, after they reached the targeted weight range, were processed. Twenty frozen carcases (five control hens, five control roosters, five trial hens and five trial roosters with live weights and average live weights matched) were analyzed for breast meat yield, meat quality and cook loss. Statistical analyses of all the data were then undertaken.

Pairs of control and trial carcases, matched for weight, were selected from the coded chickens by an independent person, and the chickens were roasted side by side in a fan-forced convection oven in separate trays (Trial 1) or in pierced oven bags (Trial 2) at 200C (Trial 1) or 175C (Trial 2). The circumference of each drumstick muscle was measured before the birds were cooked to the same degree of doneness and the meat analyzed by a tasting panel. The data for the complete feeding trial are set forth below.

**TABLE 2**

***Predicted means for the effect of Zingibain on final live-weight and carcass weight after adjustment for sex and after correction to the same initial live-weight***

| Trait (g) | Control | +Zing | Se* | Prob* |
|---|---|---|---|---|
| Final live-weight | 2223.9 | 2342.7 | 70.0 | 0.2342 |
| Plucked weight | 2095.5 | 2208.4 | 68.1 | 0.245 |
| Carcass weight | 1527.7 | 1630.2 | 48.9 | 0.1439 |

*Se standard error; Prob: probability of pairs of results being identical

The final live-weight, plucked weight and carcass weight all showed a trend for increased weight in the Zingibain supplemented group (+Zing), although only the carcass weight approached statistical significance for the small sample at P+14.4% probability. This translates to a probability of 85.6% that the effect is real.

## TABLE 3

***Predicted means for the effect of Zingibain on breast weight, pH, cook loss, drip loss, peak force and composition, after adjustment to the same carcass weight***

| Trait | Control | Zing | Se* | Prob* |
|---|---|---|---|---|
| Breast weight g | 256.3 | 272.4 | 7.1 | 0.128 |
| pH | 5.37 | 5.37 | 0.04 | 0.96 |
| Cook loss (%) | 19.8 | 18.3 | 0.01 | 0.294 |
| Drip loss (%) | 8.2 | 7.5 | 0.9 | 0.612 |
| Peak force kg (cooked) | 1.86 | 1.89 | 0.12 | 0.951 |
| Chem Fat %: | | | | |
| Breast | 8.47 | 7.93 | 0.39 | 0.342 |
| Remainder | 27.24 | 26.51 | 0.51 | 0.321 |
| Body | 23.43 | 22.41 | 0.54 | 0.203 |
| Ash (%) | 6.46 | 6.43 | 0.21 | 0.816 |
| Protein (%) | 3.93 | 4.72 | 0.37 | 0.154 |
| Dry Matter (%) | 33.7 | 33.6 | 0.46 | 0.799 |

*Se standard error; Prob: probability of pairs of results being identical

Breast weight approached statistical significance (P=0.128), with the Zingibain treatment having 16 g (6%) more breast muscle than the controls when compared at the same carcass weight. The same trends were found for the three individual muscles of the chicken breast. Using the full data set in the first of these tables, there was a trend for the Zingibain treated chickens to produce heavier carcasses than the controls. These means were then used to calculate the breast weight for Zingibain-treated and control chickens, using the regression equations calculated from the data from the 20 frozen carcasses referred to above. The predicted breast weight of a Zingibain-treated chicken with a carcass weight of 1630.2 g was 269.8 (+/- 7.1), whilst the predicted breast weight of a control chicken with a carcass weight of 1527.7 g was 236.0 g. From these data, the cumulative advantage of Zingibain treatment was estimated to be of the order of 14%.

There was no effect of Zingibain treatment on pH or shear force for the cooked breasts, reflecting the lack of collagen in chicken breast. The cook and drip loss showed a trend for lower losses in Zingibain treated samples. Chemical fat percentage was measured on the breast and the remainder of the carcass (skin off) showing a trend to lower fat in the treated chickens. Protein percentages trended to a higher percentage for the treated chickens.

**TABLE 4**

***Cooked meat taste tests***

| | **Trial 1** | | **Trial 2** | |
|---|---|---|---|---|
| Sex of chicken: | C: M | T: F | C: F | T: M |
| Drumstick circumference | C: 14.1 cm | T: 14.7 cm | 15.7 cm | T: 16.7 cm |
| Carcass weight uncooked | C: 1482 g | T: 1518 g | C: 1850 g | T: 1900 g |
| Carcass weight cooked | C: nm | T: nm | C: 1295 g | T: 1370 g |
| Carcass cook loss | C: nm | T: nm | C: 555 g | T: 530 g |
| Breast weight cooked | C: nm | T: nm | C: 310 g | T: 375 g |
| Cooking time | C: 100 min | T: 60 min | C: 90 min | T: 90 min |
| Cooked meat colour | C: off-white* | T: white* | C:pink/brown# | T: white# |
| Fat | C: fatty* | T: not fatty* | C: fatty# | T: not fatty# |
| Juiciness | C: dry* | T: juicy* | C: dry# | T: juicy# |
| Palatability preference | C: 0/7 | T: 7/7 | C: 0/4 | T: 4/4 |

nm not measured

* 7/7; #4/4

These taste test results are consistent with the trends in the above sets of data, with the Zingibain-treated chickens having a lower cook loss (7%) and more cooked breast meat (18%) for the same carcass weight, and also having whiter meat and less intra-muscular fat. The above taste testers, and other sets of taste testers for similar pairs of birds, unanimously determined that the Zingibain-treated chickens were more palatable, exhibiting less fat, more juice and whiter meat.

The consistent trends in the results across the measurements herein are of clear relevance for the chicken industry.

## EXAMPLE 10

### *Degradation of blood clots by Zingibain*

Fibrin and fibrinogen, from which fibrin is produced, have extremely important functions in animals. At the same time, however, these two proteins are associated with the occurrence of some of the more common diseases such as, for example, thrombosis.

Fibrinogen is a plasma glycoprotein with a molecular weight of 340,000 Da. It is a dimeric protein, with each monomer unit being composed of disulfide linked chains Aα, Bβ and γ, forming a dimeric tri-nodular structure (refer to Figure 4).

The α-helical coiled-coil domains of $E_5$, consisting of residues Aα50-78, Bβ85-114, and γ21-48, have an interesting structural feature. Coiled-coil sequences are usually characterized by a "heptad repeat", where every third then fourth residue is usually apolar and closely packed in the core. In $E_5$, however, there is one three-residue deletion from the heptad repeat of each chain located at homologous positions (Aα65, Bβ100 and γ36) midway along the coiled-coil domain. These deletions or 'stutters' prevent the close packing. Furthermore, in this stutter region of the Bβ chains there are prolines at position 99 where the bend occurs with arginine at $P_1$, facilitating hydrolysis by Zingibain.

The $E_5$ fragment is of relevance because it provides information about the topology of the fibrin clot. The endogenous hydrolysis of fibrinogen by thrombin at two Arg-Gly bonds liberates FpA from the two αN and FpB from the two βN chains. The liberation of the two FpA's results in the formation of two positively charged "knobs" on the E-domain consisting of Gly-Pro-Arg residues at positions 19-21 of the α-chains, which interact spontaneously with complementary "holes" pre-existing within the γ-chain C-termini on D-domains of neighbouring fibrin monomers (Hanna *et al.*, V.J. *Biochem., 23:* 4681-4687;

1984). The liberation of FpB forms a GHR knob which is thought to contribute to the association between protofibrils (Laurent and Blomback, *Acta Chem. Scand. 12:* 1875-1877; 1958; Hantgan *et al.,* "Fibrinogen structure and physiology" in "Hemostasis and Thrombosis: Basic Principles and Clinical Practice" Colman, R.W.; Hirsh, J.; Marder, V.J.; Salzman, E.W. (eds) J.B. Lippincott Company, Philadelphia, 1994, pp 277-300; Muller *et al., J. Mol. Biol. 174:* 369-384; 1984). Once the fibrin polymers are formed, they are covalently stabilized by transglutamination, a process catalysed by coagulation factor XIIIa.

The γ–γ cross-linked fibrin molecules are degraded by plasmin through hydrolysis of Lys-X and Arg-X bonds located in the coiled-coil region with one Lys-Met bond hydrolysed in the Aα-chain protuberances (Hantgan *et al.*, 1994, *supra*). Depending on the degree of cross-linking, this produces monomeric D and E domains, dimeric D-domains ("D-dimers"), the Aα-chain protuberance, Bβ1-42, Bβ15-42 and lower molecular weight peptides from within the coiled-coil region (Hantgan *et al.*, 1994, *supra*).

Fibrinogen and fibrin are rich in proline residues with hydrophilic residues at $P_1$. An SDS-PAGE electrophoresis study of the Zingibain degradation of purified human *fibrinogen* with added 2-mercaptoethanol to reduce the disulfide bridges showed that the individual α, β and γ-chains, which gave 3 bands in the region 60-52 KDa, were each totally degraded.

Furthermore, SDS-PAGE studies of Zingibain hydrolysis of human cross-linked *fibrin* with added 2-mercaptoethanol to break down the disulfide bridges (overnight incubation at room temperature) show the band from the γ-γ cross-linked chains completely removed. The dominant bands in the gel for the hydrolysed fibrin are between 40 and 50 KDa. Polymerized cross-linked fibrin forms blood clots, thereby causing thrombosis. These results demonstrate that Zingibain is able to degrade blood clots efficiently.

Further, by hydrolyzing fibrinogen and possibly other members of the blood-clotting cascade, such as prothrombin, Zingibain is able to prevent the formation of blood clots, as

shown by its ability to have significant effects on the prothrombin time assay for fresh citrated plasma, as set forth in Table 5.

**TABLE 5**

***Normal citrated clotting times by prothrombin time assay using tissue thromboplastin***

| Incubation Time (min) | 1 µg/mL Zingibain Clotting Time (sec) | 5 µg/mL Zingibain Clotting Time (Sec) |
|---|---|---|
| 0 | 14 | 14 |
| 15 | 16 | 18 |
| 30 | 32 | 46 |
| 60 | 48 | 84 |
| 120 | >200 | >200 |

These results indicate that, perhaps at levels as low as 1 ng/mL, Zingibain could limit the level of clots in blood.

**EXAMPLE 11**

***Zingibain reduces inflammation***

Fibrin and fibrinogen are closely associated with inflammation, which is defined broadly as a protective response of the body to tissue injury or destruction. Thrombin and factor XIIIa, which are generated immediately at the site of tissue damage, convert intra- and extravascular fibrinogen at the site to cross-linked fibrin. The fibrin meshwork entraps blood cells, limiting blood loss from the site and confines, to the site, inflammatory cells such as, for example, platelets, granulocytes, monocytes and lymphocytes, which would otherwise circulate.

Some of these cells as well as endothelial cells express on their outer surface cellular adhesion molecules (CAMs) that, when activated, have significant affinity for fibrin and fibrinogen. The platelet CAM, an integrin ($\alpha_{IIb}\beta_3$), recognizes the final 12 residues of the

γC-chains of fibrin and fibrinogen (Peerschke, *Semin Hematol. 22:* 241-259; 1985) and possibly the Arg-Gly-Asp sequences within fibrin and fibrinogen Aα-chains (Calvete, *Proc. Soc. Exp. Biol. Med. 208:* 346-360, 1995). Neutrophils, monocytes and lymphocytes express at least two relevant CAM's, also integrins. One, $\alpha_M\beta_2$, recognizes γ190-202 and γ377-395 within the fibrin and fibrinogen D-domains (Ugarova *et al., J. Biol. Chem. 273:* 22519-22527; 1998); the other, $\alpha_\chi\beta_2$, recognizes a Gly-Pro-Arg sequence at the fibrin αN-chains (Loike *et al., Proc. Natl. Acad. Sci. USA 88:* 1044-1048; 1991). Endothelial cells express two receptors for fibrin and fibrinogen: the integrin, $\alpha_v\beta_3$, which recognizes Arg-Gly-Asp within the fibrin and fibrinogen Aα-chain (Hawiger, "Adhesive interactions of blood cells and the vascular wall" in "Hemostasis and Thrombosis: Principles and Clinical Practice" Colman, R.W.; Hirsh, J.; Marder, V.J.; Salzman, E.W. (eds) J.B. Lippincott Company, Philadelphia, 1994, pp 762-796), and intercellular adhesion molecule 1 (ICAM-1), a member of the immunoglobulin gene superfamily, recognizes γ117-133 (Languino *et al., Proc. Natl. Acad. Sci. USA 92:* 1505-1509; 1995).

Therefore, within the inflammation site, fibrin and fibrinogen are able to adhere to a variety of cells, keeping them in the location of the inflammation. In accordance with the present invention, this effect of fibrin and fibrinogen on inflammation may be abated by the degradation of fibrin and fibrinogen by Zingibain.

## EXAMPLE 12

### *Zingibain removes skin cancers*

When Zingibain is applied topically in a cream base to human solar keratoses and to initial stages of basal cell carcinomas, the keratoses are destroyed leaving no scar and the scaly tissue from the incipient basal cell carcinomas is removed. The following four studies, and other similar experiments, provide evidence that Zingibain cream formulations may constitute a powerful, simple treatment of skin cancers.

***(a) Patient: DG; date of birth: 28/12/1912***

The Zingibain cream formulation comprised ingredients: Aqua, glycerine 10%, cetearyl alcohol 10%, *Z. officinale* root extract (Zingibain) 0.3%, mineral oil, petrolatum, ceteareth 20.

All observations on history and treatment were provided by the registered nurse in care of the patient. Over a 7-year period, three skin lesions were removed by excision. No skin lesions were noted on arms or hands. Six years later, a skin lesion on the left-hand side nose (facing) was first observed, in January/February. It commenced with a scratch from a rose bush. Another lesion on the crest and right-hand side (facing) of the nose was not obvious prior to commencement of treatment on the left-hand lesion, which began six months later, in August.

This patient's history was as follows:

*March:* lesion larger, swab taken, and lesion after treatment with antibiotics resolved to a small spot.

*May:* lesion reappeared with large reddened area and with intermittent bleeding episodes.

*June:* Checked by the dermatologist in early June, who decided to reassess in late July regarding excision and grafting.

*July:* No further growth at this stage. The dermatologist left the lesion for further assessment and treatment in view of age of patient. Raised skin area on back of right hand and a reddened area on left forearm present by late July.

Zingibain cream was applied once daily by the registered nurse to upper aspect of nose, back of right hand and left forearm. Treatment commenced in August. The lesion on the

left forearm was healed within three weeks, and the skin had attained normal tone and colour a month later. By the time the tube of cream became empty, approximately 10 weeks later, the lesion on the back of the right hand had healed with a small, hardened area still present. This hardened area had disappeared within two weeks, and the skin had assumed normal tone and colour. The lesion on the left-hand side (facing) of the nose had also healed completely by the time the tube of cream was empty, and the lesion on the crest and right-hand side (facing) only had a small area still raised.

In conclusion, a once-daily treatment of these skin cancers with Zingibain cream containing 0.3% Zingibain healed the lesions within a three month period. While a twice-daily application is usually recommended, the nurse was only able to provide the treatment once daily in this case. Nevertheless, the results were obvious.

***(b) Patient: MS; date of birth: 3/10/1930***

The Zingibain paraffin cream formulation comprised ingredients: Aqua, glycerol 9%, light liquid paraffin 9%, soft white paraffin 4.5%, *Z. officinale* root extract (Zingibain) 0.3%, methyl hydroxybenzoate 0.2%, dichlorobenzyl alcohol 0.1%.

The history of this patient was as follows:

*October:* Two solar keratoses on the side of the face were cryogenically removed by a dermatologist. However, following treatment, the wounds did not heal fully and continued to exhibit surface roughness.

*December:* The spots began to redden and increase in size. Zingibain paraffin cream was applied three times daily to the two spots.

*February:* When treatment commenced, the upper spot was approximately 3.5 mm in diameter and raised about 3 mm. The adjacent lower spot was approximately 4.5 mm in diameter and raised about 2 mm.

*Upper spot:* After one week, the upper spot began to contract and raise visibly, the surface becoming crusty and then flaking off. By the fourth week, the keratosis had disappeared, leaving healed skin.

*Lower spot:* The lower spot followed the same changes and disappeared 10 days later than the top spot, again leaving healed skin.

The dermatologist inspected the area of the spots the following March and October and found no sign of the keratoses.

***(c) Patient: FH; date of birth: 11/12/1936***

Patient FH had a history of basal cell carcinomas on his arms and legs. These were periodically removed cryogenically.

Zingibain paraffin cream was applied twice a day to some incipient basal cell carcinomas for periods of four to six weeks. The Zingibain formulation comprised the same ingredients as listed in the previous case. The red, scaly, raised patches diminished in size over the period to leave clear skin with no scaliness and with no raised patches, and with either a much lighter red colour to the skin or no redness at all.

***(d) Patient: DP; date of birth: 10/6/1953***

Patient DP had a recorded medical history of basal cell carcinomas, having had four excisions with the latest excision on the nose being unsuccessful and requiring extensive radiation therapy. Over the previous 10 years, it had been necessary to have liquid nitrogen treatment every six to 12 months for the removal of scaly skin patches and "sores" that are precursors of basal cell carcinomas.

Zingibain paraffin cream (as above) was applied twice a day for six weeks to an area on

the right-hand side of the forehead that had extensive patches of scaly skin. After treatment, there were no signs of the scaliness, and no new scaly patches have appeared since (six months) in that area.

## EXAMPLE 13

### *Zingibain aids cell harvesting*

The extracellular domains of cell membrane proteins have a range of functions including acting as the receptor molecule for a signal to the cell or as the cell adhesion molecule (CAM) for various purposes. The expression of some of these molecules, or the mutation of these molecules, is associated with particular diseases such as cancers. Although fibrin is not an integral membrane protein, it binds to cell surfaces either through physico-chemical adsorption or through binding to specific CAMs. One of Zingibain's applications is to degrade the adsorbed fibrin, so that cultured cells do not adhere to their containers and can be harvested efficiently. Trypsin is commonly used for this but, because of its more general protease activity, in addition to cleaning off the fibrin from cell surfaces, it can also cleave off desirable cell membrane proteins that are required for the cell to function.

Zingibain's much greater protease specificity allows it to be used as a replacement for trypsin. It displays efficient removal of fibrin from the cell surfaces, but with less risk to cell membrane proteins unless those proteins have suitable proline residues exposed for hydrolysis.

Like other classical cadherins, E-cadherin is a single-pass, Type I cell surface glycoprotein which mediates cell-cell adhesion. E-cadherin is the principal cadherin found in epithelial tissues; in confluent epithelial cell monolayers, E-cadherin is found concentrated in adherens junctions as well as more diffusely throughout the lateral surfaces where cells adhere to one another.

For standard tissue culture procedures, where epithelial cells (e.g. MCF-7, MDCK) must be periodically passaged, treatment with a combination of trypsin and EDTA is commonly

used to separate cells. This combination acts, at least in part, by cleaving E-cadherin and thus disrupting the cell-cell contacts. Of note, cleavage of E-cadherin by trypsin is sensitive to both trypsin activity and the extracellular calcium concentration. The cadherin ectodomain possesses calcium coordination sites and its conformation is calcium-sensitive. In the presence of calcium, the cadherin ectodomain adopts a rigid, rod-like orientation and is resistant to low concentrations of crystalline trypsin. If extracellular calcium is chelated, however, the ectodomain becomes sensitive (e.g. to 0.05% w/v crystalline trypsin).

For these experiments, it was desired to isolate individual epithelial cells, under conditions that preserve the cellular cadherin (this is quite independent of routine passaging of cells, where the cadherin is replenished). This can be quite difficult using the common cocktail of crystalline trypsin (0.05%) and calcium, at least partly because epithelial cells form calcium-independent desmosomes. Often cells are isolated only in sheets or clusters and vigorous trituration (with attendant shear damage) is necessary to separate them further. Accordingly, Zingibain was trialed as an alternative protease.

A range of Zingibain concentrations (0-5 mg/ml) was tested, diluted in Hanks balanced salt solution supplemented with 2 mM $CaCl_2$ (pH 7.4). MCF7 mammary epithelial cells (a well-differentiated breast cancer line which expresses endogenous E-cadherin) were grown to confluence and exposed to Zingibain for up to 10 min. By visual inspection, cells incubated in the higher concentrations (2-5 mg/ml) had separated by 5 min; at the lowest concentration good separation was seen by 10 min. Cells were collected by centrifugation and the total expression of E-cadherin assessed by Western blotting. No change in total E-cadherin levels was observed in any of the Zingibain-treated samples after 10 min incubation. The functional status of the cadherin was then assessed by the ability of cells to adhere and spread upon cadherin-coated substrata. For these studies, cells were isolated by exposure to 1 mg/ml Zingibain for 10 min. Adhesion and spreading of these isolated cells was excellent, suggesting that the functional competence of the cadherin was preserved.

EXAMPLE 14

Zingibain inhibits a virus

The cell-membrane proteins of viruses, such as neuraminidase and hemagglutinin of the influenza virus, are proline rich with multiple sites for hydrolysis by Zingibain. These proteins are essential for the infection process. Their cleavage inhibits the viral infection and proliferation.

An antiviral drug assay for testing the inhibitory activity of a drug against viruses was used for Zingibain with the mosquito-borne virus, Ross River Virus (RRV), at a dilution of $10^{-5}$ and $10^{-6}$. The virus was mixed with Zingibain at 0.020 mg/mL, and allowed to incubate at pH 7.2 for 2 hours. This was added to a confluent monolayer of Vero cells. The plaques produced by the virus were counted.

**TABLE 6**

***Plaque assay of Ross River Virus incubated with Zingibain (0.020 mg/mL) In Vero cells***

| RRV | Av. No. Plaques with Zingibain | Av. No. Plaques without Zingibain |
|---|---|---|
| -5 | 46 | 140 |
| -6 | 4 | 20 |

Higher concentrations of Zingibain could not be used in this type of assay, which relies on the cells being adhered to a glass surface, because Zingibain rounds up cells such as Vero cells from a surface. At 0.02 mg/mL, Zingibain inhibited RRV by up to 80%.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in

this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

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